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Fusobacterium polymorphum was examined by transmission electron microscopy. Organisms negatively stained with phosphotungstic acid showed the typical convoluted gram negative surface morphology. The same preparations revealed variability in cell length and the occasional presence of a circular polar appendage. The characteristic three layered morphology of the gram negative cell envelope was demonstrated in ultra-thin sections of these oral anaerobic bacteria. In some areas, there was an outpouching of the outer membrane which enclosed an enlarged area of the periplasmic space.

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#20: Abstract

Ruthenium red/osmium tetroxide fixed organisms displayed a layer of dense surface granules which did not appear in the thin sections of F. polymorphum that were fixed with osmium tetroxide alone. Proposed pathogenic functions of the outer membrane lined periplasmic sac and the surface polysaccharide coat are discussed.

Abstract:

Fusobacterium polymorphum was examined by transmission electron microscopy. Organisms negatively stained with phosphotungstic acid showed the typical convoluted gram negative surface morphology. The same preparations revealed variability in cell length and the occasional presence of a circular polar appendage. The characteristic three layered morphology of the gram negative cell envelope was demonstrated in ultra-thin sections of these oral anaerobic bacteria. In some areas, there was an outpouching of the outer membrane which enclosed an enlarged area of the periplasmic space. Ruthenium red/osmium tetroxide fixed organisms displayed a layer of dense surface granules which did not appear in the thin sections of *F. polymorphum* that were fixed with osmium tetroxide alone. Proposed pathogenic functions of the outer membrane lined periplasmic sac and the surface polysaccharide coat are discussed.

Introduction:

Members of the family *Bacteroidaceae* are indigenous organisms of the human oral cavity and are major pathogens in anaerobic infections of the oropharynx (21). One of these organisms, *Fusobacterium polymorphum*, has been shown to increase numerically in cases of advanced periodontitis (18,20). The pathogenicity of this and other gram negative, anaerobic, non-spore forming, filamentous bacteria is believed to result from hypersensitivity states developed by the host in response to immunogenic cellular components (5,13). Other proposed mechanisms of tissue destruction include the ability of cell wall lipopolysaccharides of the *Bacteroidaceae* to display alternate complement pathway activity (1) and the capacity to resorb bone *in vitro* (10).

Initial reports describing oral *Bacteroidaceae* by thin section microscopy (11) indicated that the cell wall of *Leptotrichia buccalis* showed a typical gram negative three layered cell envelope of a wavy outer membrane, a solid intermediate layer (peptidoglycan), and an inner cytoplasmic membrane. This report was in support of the suggestion (8) that this organism should be classified with the gram negative *Bacteroidaceae*.

A more recent ultrastructural study (16) of oral filamentous bacteria indicated that a strain of *L. buccalis* could be distinguished from other gram negative bacilli by scale-like membranous folds superficial to the external lamina of the outer membrane. In addition, this same report revealed that the inner cytoplasmic membrane and the intermediate dense layer (peptidoglycan) of the cell envelope were involved in the formation of transverse septae in *L. buccalis*. These results were consistent with similar unusual morphologic features observed in a different strain of *L. buccalis* which we have reported elsewhere (C. E. Hawley and W. A. Falkler, Jr. J. Dent. Res. 54B:IADR Abs. L-212). To date, unique morphologic features have not been demonstrated in

strains of *Fusobacterium* or *Bacteroides*.

We have been investigating the immunopathologic potential of *Fusobacterium polymorphum* in the gingival crevice and oral wounds. We felt that a morphologic examination of this organism would be a valuable adjunct to our studies. The detection of unusual morphologic features as seen with *L. buccalis*, might facilitate the identification of this organism in dental plaque and tissue fluids. Other structural details of *F. polymorphum* might be relevant to its ability to colonize the oral cavity or its pathogenicity in oral tissues.

Materials and Methods;

Growth of Microorganisms: *Fusobacterium polymorphum* (ATCC #10953) was grown under anaerobic conditions using the Gas-Pak System (Baltimore Biological Laboratories, Cockeysville, Maryland). A liquid modified tryptone media, pH 7.2, containing Bacto-tryptone, 10 g; Bacto-yeast extract, 10 g; K_2HPO_4, 1.25 g; $MgSO_4 \cdot 7H_2O$, 1.25 g; glucose, 2 g; and sodium thioglycollate, 5 g in a liter of distilled water was employed.

Electron Microscopy: Negative stained preparations of *F. polymorphum* were obtained by using 48 h broth cultures and employing the techniques of Gregory and Pirie (9). Micro-drop samples of broth cultures were placed on carbon coated and Formvar (0.25% polyvinyl formal) supported 300 mesh grids. Each drop was allowed to stand for 1 min. Excess fluid was removed by adsorption. Phosphotungstic acid (2.0% pH 7.4), with 50 µl/ml bacitracin as a wetting agent, was added with a micropipette and allowed to stand for 30 sec before the excess was removed. The air dried grids were then viewed in an aligned and calibrated Siemens Elmiskop electron microscope at an accelerating voltage of 60 kv.

F. polymorphum were also prepared for thin section electron microscopy. One ml of a 1.0% solution of osmium tetroxide (OsO_4) in veronal buffer, pH 7.2, was added to 30 ml of a 48 h broth culture. After centrifugation at 10,000 x g, the broth was decanted, and the cell pellet was washed once in veronal acetate buffer. The cell pellet was fixed for 2 h at room temperature in 1.0% OsO_4 and then washed twice in the veronal buffer. The cells were resuspended in 2.0% Noble agar at 45 C and poured onto a glass microscope slide. After the agar had cooled to room temperature, 1 mm^3 blocks were cut and placed into a post-fixation solution of 0.5% uranyl acetate in veronal buffer for 2 h at room temperature (14). Alternatively, 30 ml broth cultures were fixed with ruthenium red and osmium tetroxide following the methods of Cagle *et al.* (2). The cell pellet

formed after harvesting at 10,000 x g for 10 min was treated for 1 h at room temperature with 1 ml 0.45% ruthenium red in distilled water, 1 ml 0.2 M cacodylate buffer at pH 7.2, and 1 ml distilled water. One ml 0.45% ruthenium red, 1 ml 0.2 M cacodylate buffer, and 1 ml 25% gluteraldehyde in 0.2 M cacodylate buffer were then added, and the mixture was kept at room temperature for an additional hour. The cells were washed twice in a solution containing equal volumes of 0.45% ruthenium red, 0.2 M cacodylate buffer, and distilled water. After the washing step, the cells were fixed again for another hour in 1 ml 0.45% ruthenium red, 1 ml 0.2 M cacodylate buffer, and 1 ml 4% OsO_4 in 0.2 M cacodylate buffer. The cells were washed again in the wash solution containing ruthenium red, and then washed twice more in 0.2 M cacodylate buffer alone. The cell pellet was resuspended in 2% Noble agar at 45 C and pipetted onto a glass microscope slide. After cooling, 1 mm blocks were cut and placed into a post-fixation solution of 0.5% uranyl acetate in 0.2 M cacodylate buffer.

Both the OsO_4 and the ruthenium red/OsO_4 fixed cells were dehydrated in ethanol and embedded (23) in Spurr Low Viscosity Embedding Media (Polysciences Inc., Warrenton, Pa.). Thin sections were collected on Formvar supported 300 mesh grids, stained for 20 min in 50% ethanol saturated with uranyl acetate, and then stained for 5 min with lead citrate, pH 12 (19). The sections were washed in distilled water and examined in an aligned and calibrated Siemens Elmiskop 1A electron microscope at an accelerating voltage of 80 kv.

Results:

Whole *F. polymorphum* as prepared for examination by negative staining technique using 2.0% phosphotungstic acid were shown to be straight to slightly curved filamentous bacteria with gently tapered and rounded ends. The length of the cells varied from 7.0 μ to 9.5 μ, and the widths were always between 0.5 μ to 0.6 μ. Figure 1 shows the complex convoluted surface morphology of the cell. Pili, fimbriae, or organs of motility were not observed in any of the preparations. Frequently, however, the negatively stained cells displayed a polar, circular appendage. The creased or folded surface of these structures gave them the appearance of collapsed membranous sacs. In some preparations, the continuity between the outer membrane of the parent cell and the sac could be visualized as in Figure 2.

From the ultra-thin sections of *F. polymorphum* that had been fixed with OsO_4, without ruthenium red, details of the cell envelope were not easily visualized in resulting photomicrographs. Figure 3 indicates that a well defined wavy outer membrane can be distinguished in these preparations, but the peptidoglycan layer of the cell envelope and the cytoplasmic membrane are not well preserved for examination. The cytoplasm contains isolated packets of tightly coiled, fibrillar strands of DNA and uniformly distributed free ribosomes of approximately 100 A in diameter.

The ultra-thin sections of *F. polymorphum* that had been fixed in ruthenium red and OsO_4 are shown in Figure 4. It is apparent that the organisms in these preparations underwent less cytoplasmic shrinkage, and there was adequate preservation and visualization of the gram negative cell envelope. The outer membrane shows a wavy pattern with regions of regular periodicity of 550 to 600 A and an amplitude of 250 to 300 A. There are other areas of the 70 A wide outer membrane where the wavy pattern is absent. The intermediate peptidoglycan layer is more

electron dense, has a fine granular character, and has a uniform thickness of 90 to 100 A. The less wavy cytoplasmic membrane (70 A) is shown in intimate contact with the peptidoglycan layer and does not follow the surface undulations of the outer membrane. A surface layer of coarse granules, which are extremely electron dense in these ruthenium red/OsO_4 fixed cells, measures 90 to 100 A in thickness and is in close approximation to the external lamina of the outer membrane. This layer cannot be visualized in thin sections of the OsO_4 fixed *F. polymorphum* (Figure 3). The total thickness of the cell envelope including the surface layer is approximately 360 A.

In the same thin sections of the ruthenium red/OsO_4 fixed cells, a structure believed to be the sac observed in the negatively stained material (Figures 1 and 2) can be seen. Figure 5 shows an outer membrane lined sac at the end of the bacterium. The peptidoglycan layer and the cytoplasmic membrane of the parent cell are not involved in the structure. The ruthenium red/OsO_4 positive surface coat and outer membrane of the parent cell are extended as the lining of the apparently collapsed sac. The contents of the sac do not resemble the free ribosomes and electron dense inclusions of the cell cytoplasm.

Discussion:

The negative stained and thin section preparations of *Fusobacterium polymorphum* revealed that this organism has surface and cell wall characteristics of a gram negative bacterium. Similar findings have been reported by Takagi and Ueyama (24). We were not able to demonstrate unique morphologic features that might be utilized in the identification of *F. polymorphum* in dental plaque or wound exudates as has been suggested for *L. buccalis* by Listgarten and Lai (16).

Thin sections of the ruthenium red/OsO_4 preparations displayed a surface layer of coarse granules which did not appear in thin sections of the OsO_4 fixed cells. Ruthenium red is believed to react with monopolysaccharide and polysaccharide groups and form an electron dense reaction product (2). The finding of this histochemical marker against the surface leaflet of the outer membrane suggests the presence of cell surface carbohydrates. This outer coat may play a role in the reported specific tropism of the gram negative anaerobe for the gingival crevice (17,22) as it has been suggested by Gibbons and van Houte (6,7) that surface bacterial coats may be involved in the microbial colonization of the oral cavity through cell to surface and cell to cell attachment mechanisms. In addition, whole *F. polymorphum* cells and sonicated cellular debris have been shown to agglutinate untanned sheep red blood cells in the absence of specific antibody (W. A. Falkler, Jr. and C. E. Hawley. Infect. Immun. In Press). The agglutinating activity could be adsorbed to a variety of mammalian cell membranes. Socransky and Manganiello (22) have stressed that polysaccharide coats may be important retentive factors in maintaining the microbial ecology of the gingival crevice. The inability to demonstrate surface appendages, such as pili and fimbriae, in the negatively stained preparations of *F. polymorphum* suggests that other less obvious surface associated

mechanisms are responsible for the ability of this organism to agglutinate or adhere to cell surfaces. The retention of *F. polymorphum* in the gingival crevice *via* eucaryotic membrane adherence may be basic to the proposed role of this organism in the immunopathology of periodontal disease (18,20).

The folded and collapsed morphology of the periplasmic sac can be explained on the absence of a peptidoglycan layer in its wall. A comparison with the parent cell conformation in the same negatively stained preparations suggests that the rigid peptidoglycan layer of the gram negative cell envelope is essential in maintaining bacterial form. The significance of the outer membrane lined sac is not totally clear. Recent studies of the gram negative cell envelope indicate that there is a system of degradative hydrolytic enzymes within the periplasmic space (4). These enzymes have analogy with the lysomal enzymes of the eucaryotic cell and probably function to provide the host organism with reaction products that are of metabolic significance. Cheng *et al.* (3) have determined that one of these enzymes, alkaline phosphotase, is electrostatically associated with lipopolysaccharide components of the outer membrane. It has also been shown (15) that lipopolysaccharide-enzyme complexes can be released from growing gram negative bacteria into surrounding media. Since free floating vesicles have been observed by phase contrast microscopy in broth cultures of *F. polymorphum* (C. E. Hawley, W. A. Falkler, J. R. Mongiello, and N. K. Zeller. ASM Abs. J8, 1976), it is possible that the enlarged area of periplasm may eventually pinch off from the organism at some later time in its life cycle. If this were to occur in the gingival crevice, one could speculate on the potential damaging biologic effect such an export system for lipopolysaccharide and degradative enzymes might have on periodontal tissues.

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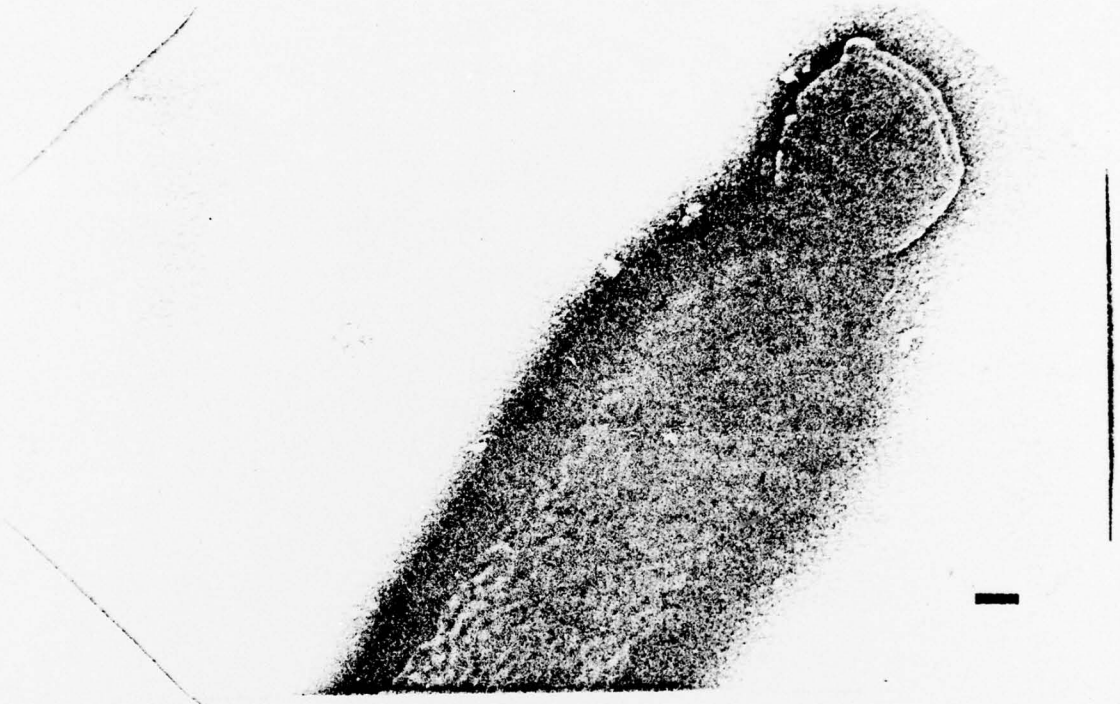

Figure 1.

Figure 1. Photomicrograph of *F. polymorphum* (ATCC #10953). Cells were negatively stained with phosphotungstic acid using bacitracin as a wetting agent and display the convoluted surface characteristic of gram negative bacteria. The polar periplasmic sac is apparent (arrow) showing surface folds. The bar represents 0.1 micron.

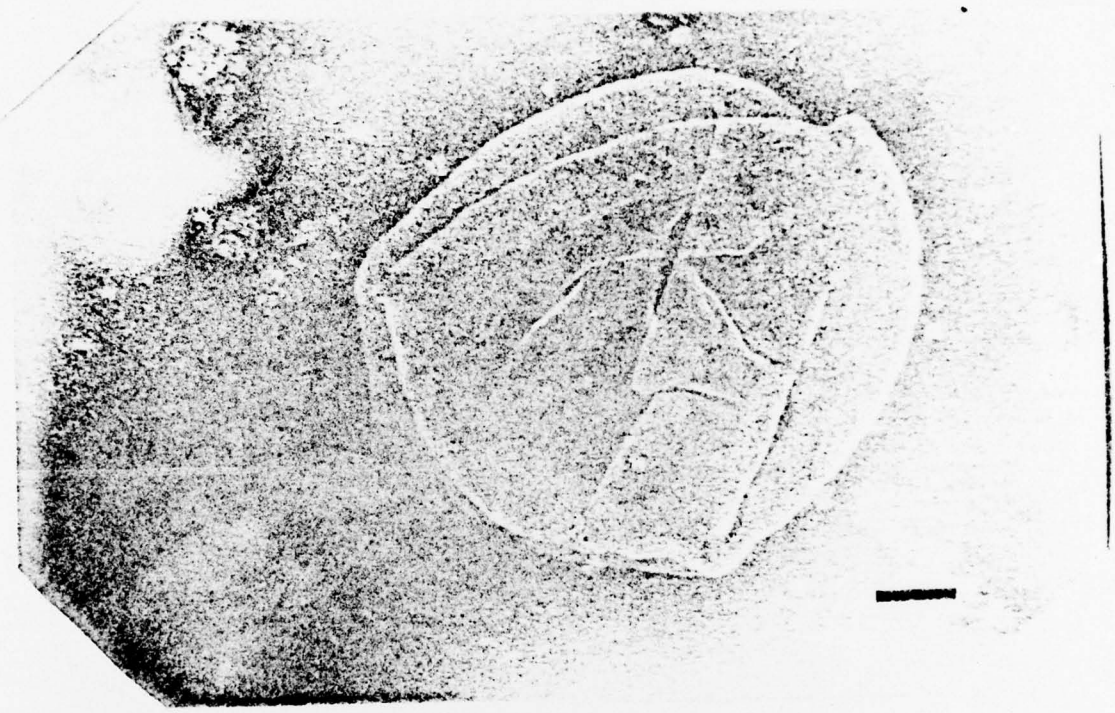

Figure 2.

Figure 2. *F. polymorphum* (ATCC #10953) negatively stained with phosphotungstic acid showing another polar sac. Arrows designate region of outer membrane continuity between the sac and the parent cell. The bar represents 0.1 micron.

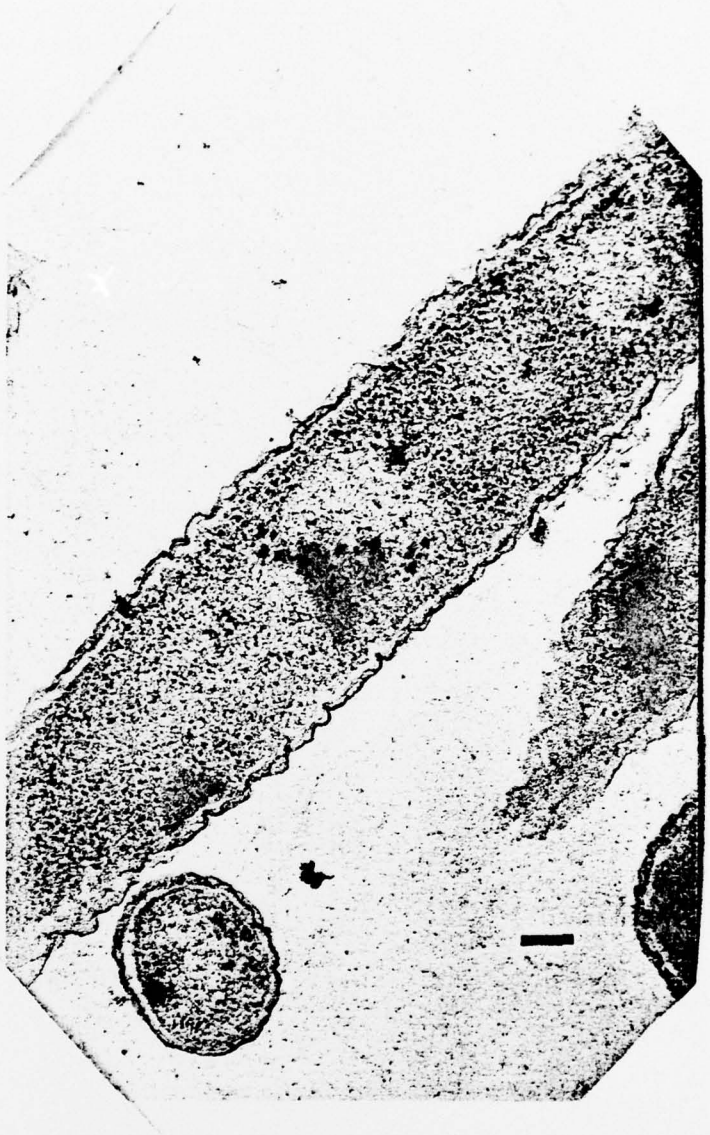

Figure 3.

Figure 3. An electron micrograph of an ultra-thin section of *F. polymorphum* fixed with OsO_4. The wavy morphology of the outer membrane (arrows) is the most apparent component of the gram negative cell envelope. The bar represents 0.1 micron.

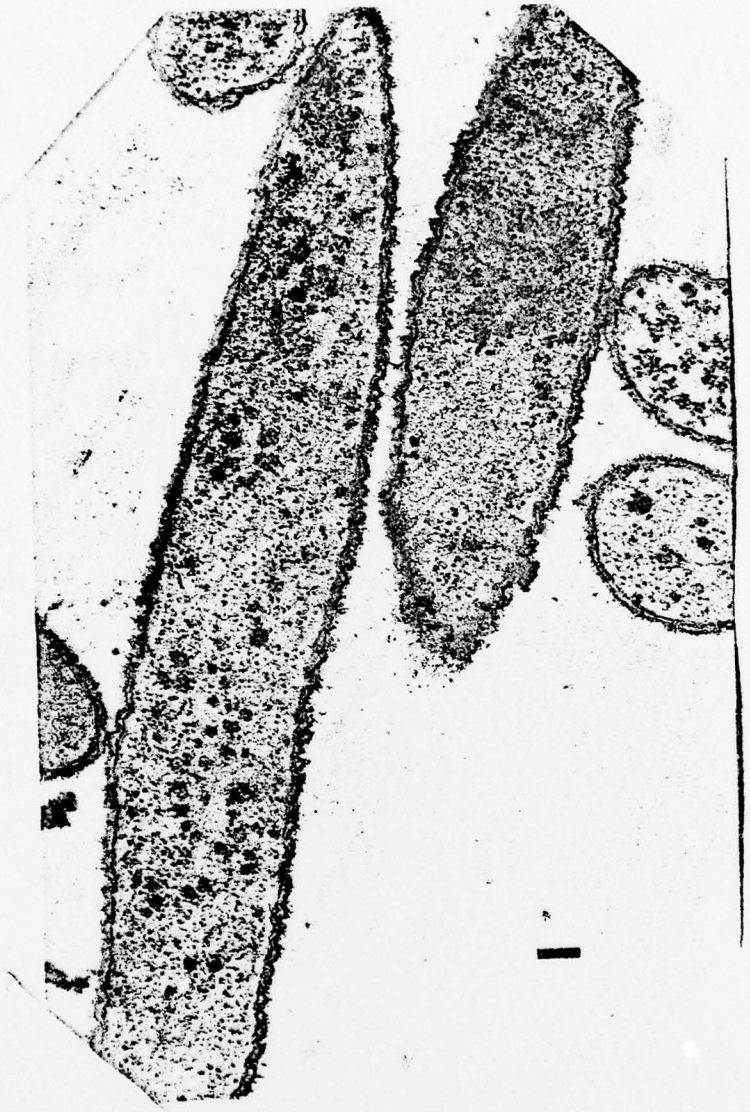

Figure 4.

Figure 4. Photomicrograph of an ultra-thin section of ruthenium red and OsO_4 fixed *F. polymorphum*. The three layers of the gram negative cell envelope are shown. A layer of electron dense granules is apparent external to the outer membrane (arrow). The bar represents 0.1 micron.

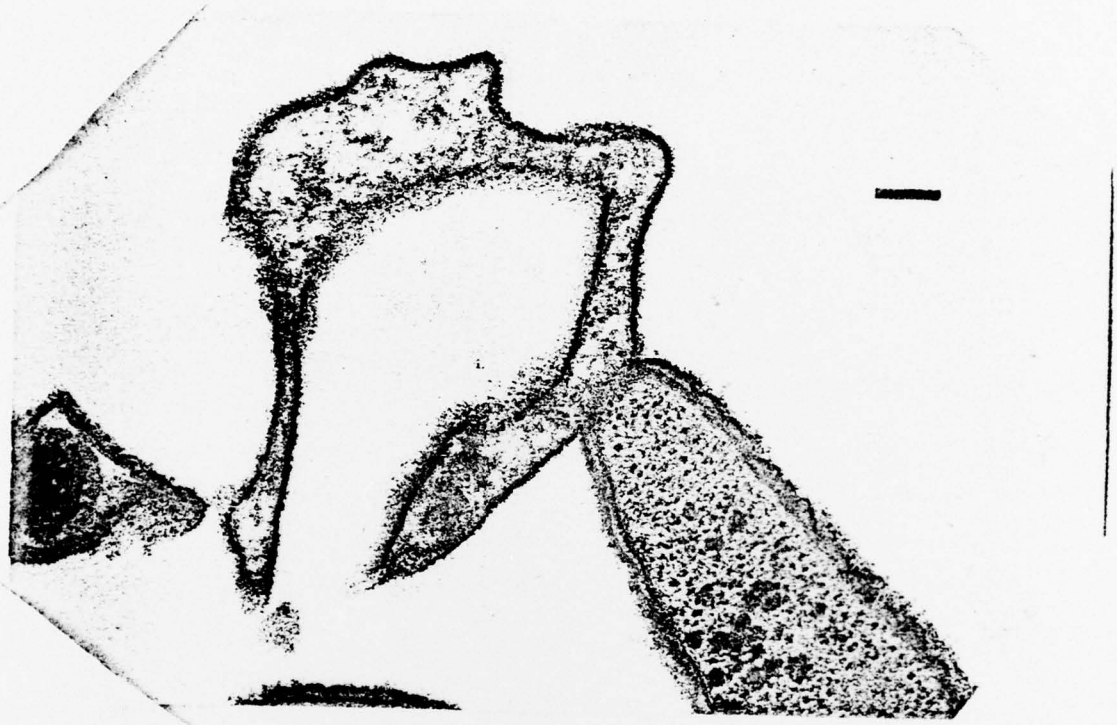

Figure 5.

Figure 5. A thin section photomicrograph of ruthenium red and OsO_4 fixed *F. polymorphum*. An outer membrane lined sac is seen at the end of the bacterium. This enlarged area of the periplasmic space is free of cytoplasmic material. The bar represents 0.1 micron.